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Original Paper

A Clinically Applicable Assay for Tumoral Thymidylate Synthase Combining Reverse Transcriptase–PCR and High-performance Liquid Chromatography

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The main clinically relevant cellular target of 5-fluorouracil (5-FU) is the enzyme thymidylate synthase (TS). Both preclinical data and clinical data in digestive tract cancer indicate that an increased amount of TS in tumours can predict for 5-FU resistance. We developed an automated method combining the principle of RT–PCR coupled with HPLC separation and quantification. The RT–PCR HPLC method was applied to *TS* determination in tumoral biopsies from patients with colorectal cancer. The PCR samples were separated and quantified using a polystyrene divinylbenzene C 18 column. Within 22 min, it was possible to elute 18 peaks representing DNA sizes ranging from 34 to 622 bp. Both separation and quantification of *β2 microglobulin* (*β2m*, internal standard) and *TS* PCR products were achieved in approximately 10 min per sample. Validation of the RT–PCR HPLC method was established by comparing RT–PCR quantification of *TS* after electrophoresis and HPLC and by comparing the RT–PCR quantification of *TS* after HPLC with the classical biochemical method. The proposed HPLC method offers a 10–50-fold sensitivity advantage over electrophoresis. In addition, this RT–PCR HPLC procedure allows not only the quantification of *TS* expression but also the direct collection of unaltered amplified DNA sequence which could be useful for sequencing analysis, since *TS* mutations have been described. The present RT–PCR HPLC method for determining *TS* expression in tumoral biopsies is a valuable analytical approach as it is specific, sensitive and clinically applicable. © **1998 Elsevier Science Ltd.**

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INTRODUCTION

5-FLUOROURACIL (5-FU) is the most frequently used antineoplastic agent for the treatment of digestive tract cancer and also plays a central role during induction chemotherapy for advanced head and neck cancer. Cellular targets for 5-FU are multiple [1] and so the resistance mechanisms responsible for treatment failure may also be numerous and complex [2]. Nevertheless, the main clinically relevant cellular target of 5-FU is the enzyme thymidylate synthase (TS). Yet, both preclinical data [3, 5] and clinical data in digestive tract cancer [6, 10] have indicated that an increased amount of TS in tumours can predict 5-FU resistance. In addition, it has been shown that tumoral TS expression is not only linked to 5-FU treatment response but also to patient survival [9, 11].

Owing to the clinical relevance of tumoral TS determination, different analytical approaches have been developed for measuring tumoral TS activity. Quantitative methods for TS determination are related to TS catalytic activity [12] or the level of 5-fluorodeoxyuridine 5′-monophosphate (FdUMP) binding [13]. However, these methods require a relatively large amount of biological material. Semiquantitative methods are based on the use of TS-related antibodies for immunohistochemical analysis [11] or, as recently described, on the application of reverse transcriptase–polymerase chain reaction (RT–PCR) methods [10, 14, 15]. More precisely, this latter analytical approach is interesting because it requires only a small tumour tissue sample (10–15 mg).

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However, classical post-PCR steps involve electrophoretic separation and precise quantification of electrophoretic bands. This is a serious limitation of the routine use of this method in determining TS in large series of tumour samples. Facing the difficulty of using conventional RT–PCR methods in a large-scale biological analysis, some authors have proposed innovative approaches for improving post-PCR separation and quantification such as capillary electrophoresis which has been successfully applied to HIV-1 virion quantification [16]. Other authors [17–22] have described high-performance liquid chromatography (HPLC)-based procedures allowing separation and quantification of PCR products by liquid chromatography. Because HPLC has become a widely accessible and widely used laboratory technique, we developed an automated method combining the principle of RT–PCR coupled with HPLC separation and quantification. The RT–PCR HPLC method has been applied and validated for TS determination in tumoral biopsies from a group of patients with colorectal cancer.

MATERIALS AND METHODS

Chemicals

HPLC gradient-grade acetonitrile was obtained from Merck (Darmstadt, Germany). Buffers were prepared using a stock solution of 1 M HPLC-grade triethylammonium acetate (TEAA, Fluka, Buchs, Switzerland). Tris, boric acid and EDTA were purchased from Sigma chimie (L'isle d'Abeau Chesnes, France).

Tumour samples

Twelve tumour specimens (average weight 100 mg) were obtained by surgical excision from 12 patients with primary colorectal cancer (6 men, 6 women; mean age 64.1 years, range 41–74 years). None of these patients had received anticancer treatment before surgical excision of the tumour. These biological specimens were stored in liquid nitrogen, then pulverised, and the tissue powder was stored in liquid nitrogen until the assay. On the day of the assay, the resulting powder was divided into two equal parts. One part was used for the TS biochemical assay and the other for the RT–PCR assay.

Extraction of RNA

Total RNA isolation was performed by using the RNA Now extraction kit (Biogentex, distributed by Ozyme, Montigny-Le-Bretonneux, France). Briefly, 50 mg of pulverised tumour were homogenised with 1 ml of kit reagent. Following the addition of 0.1 ml of chloroform and centrifugation (12 000*g*, 10 min at 4°C), the aqueous phase was precipitated with an equal volume of isopropanol. The pellet was then washed twice with 75% ethanol and resuspended in 50 µl of diethyl pyrocarbonate (DEPC) water. RNA quality was analysed by checking the presence of ribosomal RNA bands (18 S and 28 S) after electrophoresis on a 1% agarose gel.

Reverse transcription

10 µg of total RNA were pre-incubated for 5 min at 65°C in a 100 µl final volume of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1.25 mM of each deoxynucleotide triphosphate (dNTP) and 500 pmol of random hexanucleotide primers (Amersham, Les Ulis, France). One thousand units of MMLV reverse transcriptase (Gibco Life Technologies, Eragny, France) and 300 U of human placenta ribonuclease inhibitor (Amersham) were then added and the mixture was incubated for 30 min at 42°C followed by 5 min at 94°C to stop the reaction. Samples were then stored at −20°C.

PCR conditions

PCR was performed in a 0.5 ml tube by adding 10 µl of cDNA (1 µg RNA equivalent) to a total volume of 100 µl of 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 250 µM of each dNTP, 0.5 µM of each primer, 2.5 U Taq DNA Polymerase (Boehringer, Meylan, France). The sequence of the primers used for amplification of the *beta-2 microglobulin* (*β2m*, internal standard, 5′-CATCCAGCGTACTCCAAAGA-3′ (nt 97-116) and 5′-GACAAGTCTGAATGCTCCAC-3′ (nt 242–261)) as well as the *TS* gene (5′-AAACGTGTGTTCTGGAAGGG-3′ (nt 229–248) and 5′-CCATATCTCTGTATTCTGCC-3′ (nt 429–448)) allow genomic DNA to be discriminated from cDNA due to their position flanking an intron.

Amplification of tumour cDNAs was carried out for 21 cycles using a thermocycler (Perkin-Elmer 480, Norwalk, Connecticut, U.S.A.): denaturation step for 30 s at 94°C, annealing step for 1 min at 58°C and elongation step for 30 s at 72°C. Samples were then stored at −20°C.

Quantification of PCR products

Gel electrophoresis. PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide. The gel was photographed and a negative polaroid was used to quantify signal intensity by densitometry after scanning (Agfa studio scan II). The results were analysed using the NIH Image 1.45 freeware.

HPLC. The PCR samples were separated and quantified using a polystyrene divinylbenzene (PS-DVB) C 18 column 50×4.6 mm (Sarasep NPC 18 M, Santa Clara, U.S.A.) protected by a guard column filled with a 0.2 µM filter (Sarasep). The HPLC system consisted of two pumps (Waters 510, St Quentin en Yvelynes, France), an autosampler (Waters 717 refrigerated at 4°C), a column oven (Fiatron CH30 at 50°C), a variable-wavelength UV monitor (Waters 484) and a PC-based data system (Millenium, Waters). This software performs gradients and monitors chromatograms.

The aqueous buffer was 0.1 M triethylammonium acetate (TEAA) acting as a counter-ion, pH 7 (buffer A). Buffer B was prepared as a 25% solution of acetonitrile in buffer A. The flow rate of the HPLC system was 1 ml/min. Injection volumes ranged between 10 and 60 µl. The chromatographic steps were set as previously described by Oefner and associates [22] and involved a 30 min linear gradient from 40% buffer B/60% buffer A to 80% buffer B/20% buffer A. Initial conditions were obtained from the following steps: 4 min linear gradient from 35% buffer B/65% buffer A to 55% buffer B/45% buffer A, then 6 min linear gradient to 65% buffer B/35% buffer A, then 1 min linear gradient to 100% buffer B, then 100% buffer B for 3 min followed by a linear gradient during 1 min from 100% to 35% buffer B/65% buffer A which are the initial conditions of column equilibration. The UV detector was set at 254 nm. Intra-assay reproducibility testing was done by repeated injections ($n = 8$), in the same experiment, of three different PCR products obtained from a 'low TS' activity tumour (TS biochemical activity = 56 fmol/min/mg prot), a 'medium TS' activity tumour (TS biochemical activity = 200 fmol/min/mg prot) and a 'high TS' activity tumour (TS biochemical activity = 2565 fmol/min/mg prot).

Interassay reproducibility testing was done by serial injections ($n = 6$), during repeated experiments, of PCR products from the 'medium TS activity' tumour and from the 'high TS activity' tumour.

DNA standards

DNA standards were used so as to determine the resolution of the PCR–HPLC method. The DNA sizing marker was the *pBR322* DNA *Msp*I digest (Ozyme) containing 18 fragments ranging from 34 to 622 bp (used at 20 ng/µl).

The *TS* (220 bp) and *β2m* (165 bp) RT–PCR products of a given tumour were used to determine the detection threshold and the linearity of the RT–PCR method. The native RT–PCR products contained 10 ng/10 µl for *TS* and 70 ng/10 µl for *β2m* (quantified after parallel electrophoresis with *Eco*147I and *Pvu*I digested pMLX DNA–MBI fermentas, Vilnius, Lithuania).

Determination of TS activity

Pulverised tumour tissue (50 mg on average) was homogenised in 10 volumes of a 10 mM Tris-HCl buffer pH 7.4, containing 1 mM EDTA, 0.5 mM dithiothreitol and 10 mM sodium molybdate. The homogenates were centrifuged for 1 h at 105 000*g* (+4°C) and the supernatant (cytosol) used for protein determination and enzymatic assay. Proteins were measured using Coomassie G-250 (Protein Assay Reagent, Biorad Ltd., Paris) with human albumin as standard.

Tumoral TS activity was determined by a biochemical method according to Spears and associates [12] which is routinely used in our laboratory [4]. The assay was performed at 1 µmol/l ^{3}H-dUMP instead of 40 nmol/l as previously described by Spears and associates [12]. The assay consisted of incubating 25 µl of cytosol with ^{3}H-dUMP (1 µmol/l final concentration) and 5–10 methylene tetrahydrofolate (0.62 mmol/l final concentration) in a total volume of 55 µl (Tris-HCl 50 mmol/l, dithiothreitol 2 mmol/l, pH 7.3). After 10, 20, and 30 min of incubation at +37°C, the reaction was stopped on an ice bed. The excess of ^{3}H-dUMP was removed by adding 300 µl of activated charcoal (15%) containing 4% trichloracetic acid (5-min centrifugation at 14 000*g*, room temperature). The tritiated water (^3H_2O) formed during the incubation was then counted in an aliquot of 150 µl of the previous supernatant. Results were expressed as femtomoles of ^3H_2O formed per minute per milligram protein, based on the linear regression obtained from the incubation times. Each incubation time was performed in triplicate. The sensitivity limit was 10 fmol/min/mg prot. The stability of TS activity during storage, evaluated through the interassay reproducibility over eight assays (pooled cytosol), gave a coefficient of variation of 14.9%.

RESULTS

RT–PCR HPLC method performances

Figure 1 illustrates the performance of the HPLC method allowing an optimal separation of the DNA sizing markers. Within 22 min, it was possible to elute 18 peaks representing DNA sizes ranging from 34 to 622 bp. It must be emphasised that it was possible to separate correctly peaks with 10 bp difference (180–190 bp). Under these conditions one can consider that the resolution limit is 5 bp for fragments having sizes ranging from 150 to 300 bp. The PCR products obtained after co-amplification of *TS* and *β2m* from a tumoral sample were compared after gel electrophoresis or HPLC (Figure 2). Figure 2(a) shows the 165 and 220 bp

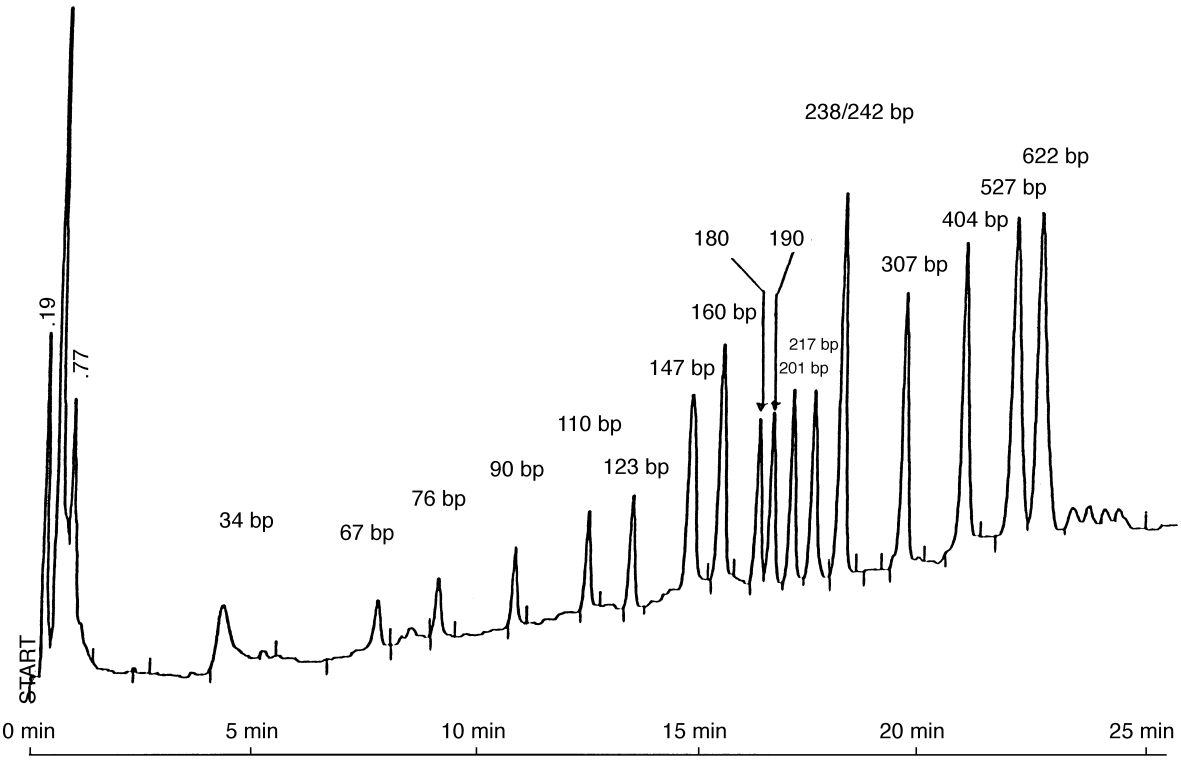

Figure 1. High-resolution liquid chromatographic separation of a DNA sizing marker (*pBR 322* DNA *Msp*I digest). Column: PS-DVB-C18, 50×4.6 mm I.D. Mobile phase: buffer A: 0.1 M TEAA, pH 7.0; buffer B: 0.1 M TEAA, 25% acetonitrile, pH 7.0. Linear gradient from 60% A/40% B to 20% A/80% B in 30 min. Flow rate: 1 ml/min, temperature 50°C, detection UV at 254 nm.

DNA fragments corresponding to *β2m* and *TS*, respectively. The estimated quantities of DNA for *TS* and *β*2 were, respectively, 10 and 70 ng. For *TS*, this corresponds with 2–5-fold of the limit of detection for ethidium bromide stained gels. A 50-fold dilution of this sample was then analysed by HPLC. The results are shown in Figure 2(b). Retention times for *β2m* and *TS* were 8.5 and 9.5 min, respectively. Under these conditions, the peak for *TS* corresponded to 0.2 ng of DNA, which is twice the sensitivity limit with HPLC detection (0.1 ng). Figure 3(a) and (b) depict the linearity of the HPLC signal for *TS* and *β2m*, respectively, obtained after serial dilution of an RT–PCR product from a colorectal tumour. In each case the data points fitted a linear regression ($r^2 = 0.979$ for TS and $r^2 = 0.995$ for *β2m*).

Intra and interassay reproducibility evaluations are given in Table 1. In all cases, the coefficient of variation was below the 10% limit.

Validation and clinical applicability of the RT–PCR HPLC method

This was established by comparing RT–PCR quantification of *TS* after electrophoresis and HPLC (Figure 4a) and by contrasting the RT-PCR quantification of *TS* after HPLC with the classical biochemical method. All experiments were based on the study of a group of tumour samples from colorectal cancer patients. For these samples ($n = 12$), *TS*/*β2m*

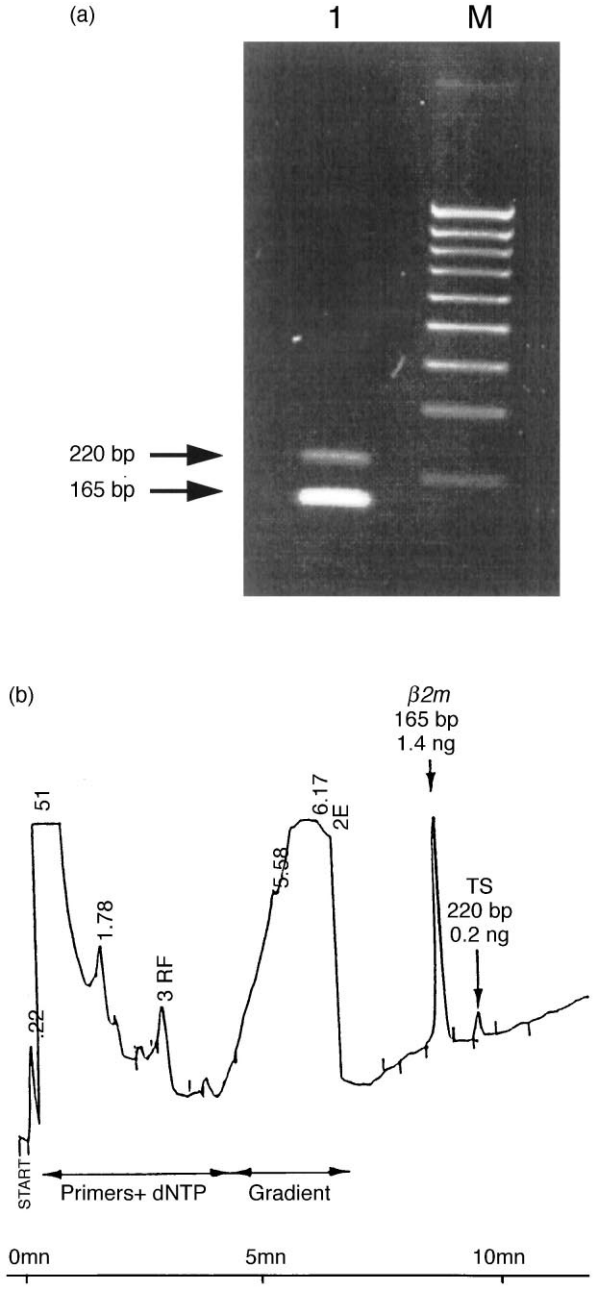

Figure 2. (a) PCR products analysis by gel electrophoresis with ethidium bromide staining after co-amplification of a tumoral sample. Lane 1: 10 µl of *TS* (220 bp) and *β2m* (165 bp) PCR products. Lane M: molecular weight DNA marker. (b) High-performance liquid chromatographic analysis of a 50-fold dilution of PCR products from the same tumoral sample (10 µl injected). Column: PS-DVB-C18, 50×4.6 mm I.D. Mobile phase: buffer A: 0.1 M TEAA, pH 7.0, buffer B: 0.1 M TEAA, 25% acetonitrile, pH 7.0. 4 min linear gradient from 65% A/35% B to 45% A/55% B, then 6 min linear gradient to 35% A/65% B, then 1 min to 100% B (3 min) followed by a 1 min linear gradient to 65% A/35% B which are the initial conditions. Flow rate: 1 ml/min, temperature 50°C, detection UV at 254 nm.

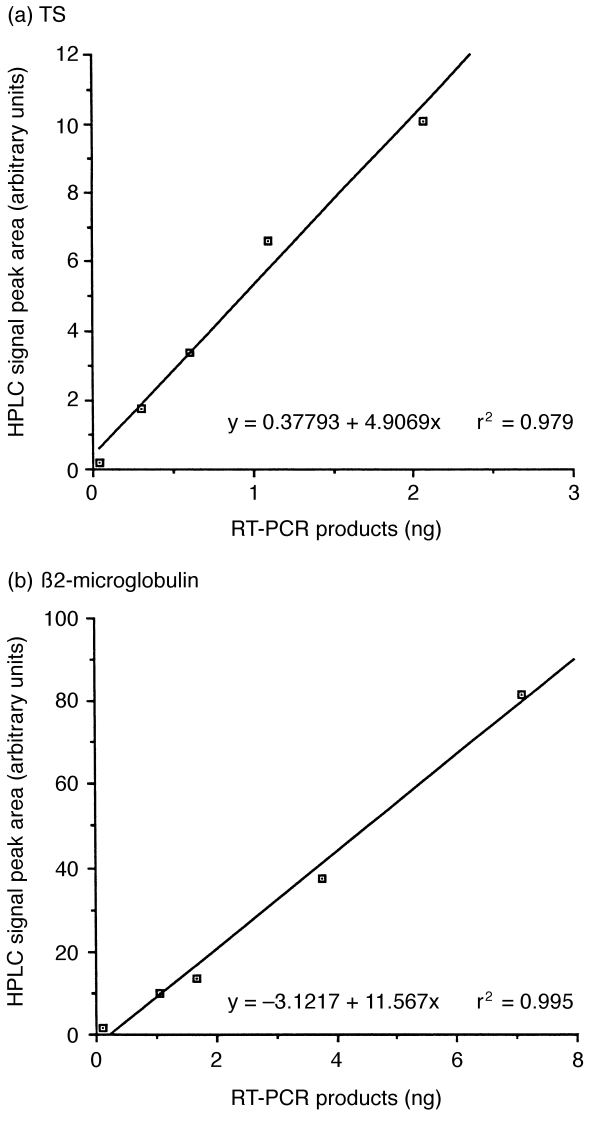

Figure 3. Linearity of the detection of the HPLC signals obtained after serial dilutions of an RT–PCR product. (a) *TS*; (b) *β2 microglobulin*.

Table 1. *Reproducibility of the RT–PCR HPLC method (coefficient of variation)*

Intra-assay reproducibility			Interassay reproducibility	
"low TS" ($n = 8$)	"median TS" ($n = 8$)	"high TS" ($n = 8$)	"median TS" ($n = 6$)	"high TS" ($n = 6$)
8.8%	3.9%	2.7%	5.2%	5.2%

For experimental conditions, see details in Materials and Methods. Reproducibility analysis was based on calculation of the peak area.

ratios ranged from 0 to 0.222 (median = 0.068) by RT–PCR electrophoresis, 0–0.250 (median = 0.050) by RT–PCR HPLC and 0–2565 fmol/min/mg prot (median = 358) by the biochemical method (TS activity). Figure 4(a) shows the good correlation between RT–PCR electrophoresis and RT–PCR HPLC ($r^2 = 0.78$). There was also a satisfactory correlation between *TS* measured by RT–PCR HPLC and TS determined by the biochemical method (Figure 4b).

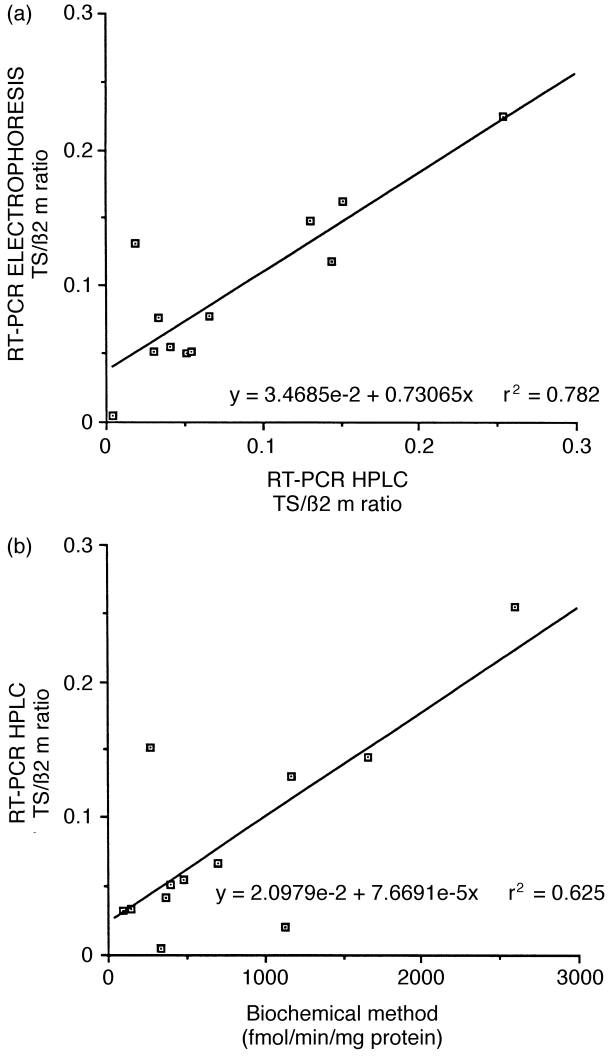

Figure 4. (a) Comparison between two methods of quantification of the expression of the *TS* gene: electrophoresis versus HPLC in 12 colorectal tumours. (b) Correlation between *TS* gene expression determined by RT–PCR HPLC and the TS protein content measured by a biochemical method (see Materials and Methods) in 12 colorectal tumours.

DISCUSSION

There is an accumulation of recent preclinical and clinical data indicating that determination of tumoral *TS* can be of value for predicting resistance to 5-FU-based chemotherapy [3–10]. Large-scale, prospective clinical investigations are thus justified for confirming the clinical value of pretreatment *TS* determination, mostly in digestive tract cancer as recently stressed by Johnston and associates [10]. Moreover, a new generation of TS inhibitors has entered clinical trials and proven their antitumour efficacy [23]. Alternatively, new drugs acting on cellular targets other than TS have shown therapeutic efficacy in metastatic colorectal cancer. Thus, *TS* tumoral determination can serve for the rational choice of an optimal chemotherapeutic regimen at the individual level. It follows that measurement of *TS* tumoral expression could become of routine use. Because TS activity varies greatly between tumour samples and its determination must frequently be done in small tumoral biopsies [14], an optimal analytical method for *TS* measurement must be both sensitive and routinely applicable. The present study provides an original approach for *TS* determination in biological samples which can fulfil these criteria. The RT–PCR-based method for *TS* determination has been previously clinically validated [10, 14, 15]. However, conventional methods for the analysis of PCR products typically involve the use of agarose gel electrophoresis followed by densitometric scanning. HPLC represents an alternative to this technique and allows, 10 min after injection of amplification products, to obtain directly the area of each peak. The use of HPLC to quantify PCR products was based on recent demonstrations of reverse-phase HPLC on non-porous PS-DVB particles in combination with ion-pair formation which are very effective for the separation of small DNA fragments [17–22]. The combined RT–PCR HPLC approach for tumoral TS determination was described and validated on both qualitative and quantitative criteria with intra- and interassay reproducibilities being satisfactory (coefficients of variation lower than 10%). We did not observe a significant reduction of the chromatographic separation performances in up to 300 injections. This is in agreement with the experience of Oefner and colleagues [22]. The limit of sensitivity was reached at 0.1 ng of *TS* PCR product injected. A minimum of 1–5 ng of DNA can be detected by ethidium bromide staining and direct examination of the gel under UV [18]. Thus, this new application offers a 10–50-fold sensitivity advantage over electrophoresis. Another advantage comes from the rapidity of the analysis since *β2m* and *TS* peaks were resolved within 15 min (including column re-equilibration time), allowing 60 samples to be run during the night with an automatic injector. The main purpose of the present work was not to explore a large number of cancer patients for their *TS* tumour expression. However, as a complement to its inherent technical aspects, the clinical applicability of the method was evaluated by exploring *TS* expression in tumour samples of human colorectal origin. It appeared that RT–PCR electrophoresis and RT–PCR HPLC correlated very well ($r^2 = 0.782$, $n = 12$). Moreover, a comparison of the same biological samples between the RT–PCR HPLC method and conventional biochemical analysis of TS activity indicated a very satisfactory concordance between these two analytical approaches. Of importance, it must be emphasised that this RT–PCR HPLC procedure allows not only *TS* expression to be quantified but also makes possible direct collection of the unaltered amplified

DNA sequence which could be useful for sequencing analysis since *TS* mutations have been recently described [24].

In conclusion, the RT–PCR HPLC method presently described for the determination of *TS* expression in tumoral biopsies is a valuable analytical approach as it is specific, sensitive and clinically applicable. This method offers an interesting tool to investigators who wish to include tumoral *TS* determination in future clinical trials.

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